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Molecular Analysis of *tet*(W) Gene-Mediated Tetracycline Resistance in Dominant Intestinal *Bifidobacterium* Species from Healthy Humans▽

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***tet*(W) was found responsible for tetracycline resistance (MICs, 4 to ≥32 μg ml^{-1}) in dominant bifidobacterial species from the gastrointestinal tracts of healthy humans. The gene from *Bifidobacterium longum* H66 proved to be identical over a 2.6-kbp region to the recently described *tet*(W) determinant of *Butyrivibrio fibrisolvens*.**

The commensal intestinal microbiota of humans and animals may act as a reservoir of antibiotic resistance genes that could ultimately be transferred to pathogens (15, 18); in fact, gene transfer between bacterial species in the gastrointestinal tracts (GIT) of mammals is known to occur (11). Bifidobacteria are among the dominant populations of the human GIT microbiota, where they are thought to play a pivotal role in maintaining the microbial balance necessary for intestinal health (19). Bifidobacterial strains are therefore frequently used as probiotics in the prophylaxis and therapy of GIT disorders (12). This practice, however, requires that they be screened for acquired antibiotic resistance determinants if the latter are not to be propagated through the food chain (14, 15, 18).

Tetracyclines inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the bacterial ribosome (2). The broad-spectrum antimicrobial properties of these agents, the absence of major adverse side effects, and their low price have led to their intensive use not only in the treatment of human and animal infections but also as prophylactic agents and growth promoters in livestock raising and aquaculture (2). This extensive use has promoted the appearance of resistance and its spread by horizontal acquisition (2, 13). Resistance is mediated through efflux proteins, ribosomal protection proteins, or antibiotic-inactivating enzymes (for a review, see reference 13).

The *tet*(W) gene, which encodes a ribosomal protection protein, has recently been described for a wide range of gram-positive and gram-negative bacteria (1, 2), including bifidobacteria (7, 10, 17). This gene has been associated with a conjugative transposon (Tn*B1230*) in *Butyrivibrio fibrisolvens* (9) that has been shown to transfer at high frequencies (up to 5.1×10^{-3} transconjugants per recipient) (16).

In a recent survey, a microbroth assay showed atypical (≥16 μg ml^{-1}) MICs of tetracycline for human bifidobacterial strains isolated from fecal samples of healthy adults without a recent history of antibiotic treatment (4). The object of this study was to investigate the nature and molecular organization of the tetracycline determinant encoding this resistance.

MICs of tetracycline for bifidobacteria. The MICs of tetracycline for a series of susceptible (14 isolates) and resistant (16 isolates) bifidobacterial species which had been assayed by microdilution (4) were precisely determined by Etest (AB Biodisk, Solna, Sweden). Assays were performed in LSM test agar (6) (90% IsoSensitest, 10% MRS, 1.5% agar; all from Oxoid, New Hampshire, United Kingdom) supplemented with 0.3 g of cysteine liter^{-1}. Plates were incubated at 37°C for 48 h in an anaerobic chamber (atmosphere, 10% H_2–10% CO_2–80% N_2). The MICs for susceptible isolates ranged from 0.25 to 1 μg ml^{-1}, whereas those for isolates thought to be resistant ranged from 4 to 32 μg ml^{-1}. MICs obtained for the same strains in duplicate experiments never exceeded 1 order of dilution.

To exclude the analysis of replicates, resistant and susceptible isolates were typed by rapid amplification of polymorphic DNA (RAPD) analysis by PCR. Amplifications were performed using primer OPA18 and PCR conditions described elsewhere (8). Seven different RAPD-PCR profiles identifying distinct strains were obtained among the resistant isolates, as follows: *Bifidobacterium longum* (11 isolates, 5 strains), *Bifidobacterium bifidum* (5 isolates, 1 strain), and *Bifidobacterium animalis* subsp. *animalis* (1 isolate, 1 strain).

Amplification and analysis of tetracycline resistance genes. The presence of genes encoding ribosomal protection proteins was checked among resistant and susceptible strains by PCR with two pairs of degenerate primers: DI–DII (3) and Tet1–Tet2 (1). Resistant strains all yielded a single PCR product of the same size when either of the primer pairs was used. Surprisingly, positive amplification was also obtained with DNA from the susceptible strain *B. longum* M21. Additional PCR assays were then performed with primers TetWF and Tet2, specific for *tet*(W) (17), and primers DI and TetMR, specific for *tet*(M) (5). No DNA was amplified from either resistant or susceptible isolates with primers for *tet*(M). In contrast, a product of about 1,250 bp was obtained for all 16 resistant isolates when primers for *tet*(W) were used. Purified amplicons were sequenced and the resulting sequences analyzed and compared to others held in public databases.

Analysis of the nucleotide sequences showed them to share

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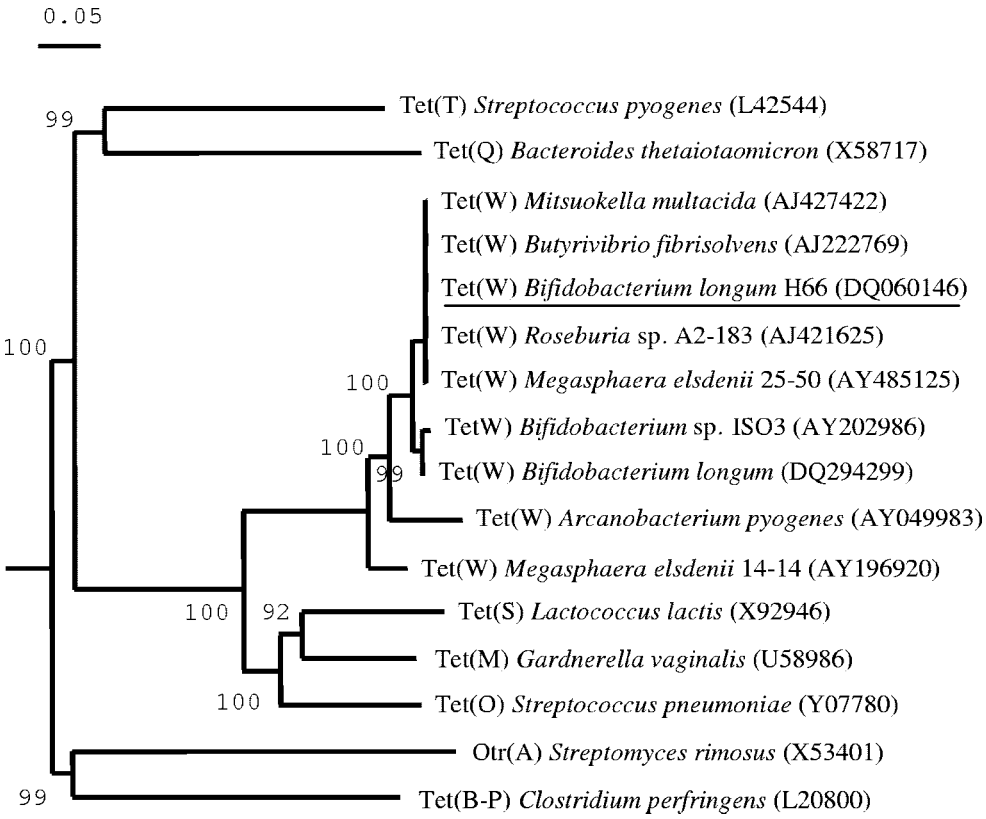

FIG. 1. Phylogenetic tree showing the evolutionary relationships of complete Tet(W) and other ribosome protection proteins from tetracycline-resistant bacteria. GenBank accession numbers for the nucleotide sequences used to derive amino acid sequences are given in parentheses. The rooted neighbor-joining tree was generated using DNAman software (Lynnon Corporation, Vaudreuil-Dorion, Quebec, Canada). Numbers beside nodes indicate bootstrap values when these are greater than 95% (based on 500 trials). Bar, 0.05% difference in amino acid sequence.

>99.8% identity. Comparison of the sequences showed a high degree of similarity at the nucleotide level to several tetracycline resistance genes. In particular, complete identity was seen with an internal segment of the *tet*(W) gene from *B. fibrisolvens* (1). Based on this homology, primers TetWSacF (5′-CCCTG GAGCTCATGCTCATGTAC-3′) and TetWSacR (5′-CCAT CGGAGCTCCATAACTTCTG-3′) were designed to amplify the whole gene and its surrounding promoter and terminator regions. Positive amplification was obtained only when DNA from the resistant strains was used as a template, suggesting that the susceptible strain may harbor a shortened (nonfunctional) version of the gene.

The amplicon was cloned into pUC19 and introduced into *Escherichia coli*, which became resistant to tetracycline (MIC, 64 μg ml^{-1}). The insert was then double-strand sequenced. At the nucleotide level, analysis of a 2,589-bp segment showed that the sequence was identical to that of *B. fibrisolvens* 1.230 (1, 9), except for a single oligonucleotide change in the coding region leading to a conservative amino acid replacement in its deduced Tet(W) protein. Further, the *B. longum* H66 sequence was found to share around 98% identity to recently reported *tet*(W) genes from bifidobacterial species (GenBank accession no. AF202986) (7, 17). A phylogenetic tree showing the relationships of the deduced Tet(W) proteins and those of other ribosome protection proteins is depicted in Fig. 1. The presence of resistance genes with nearly identical nucleotide and protein sequences in unrelated bacterial and bifidobacterial species suggests that recent horizontal transfer has occurred (1, 16). Intriguing is the fact that different MICs are repeatedly reported for identical genes (7, 10, 17). Multiple loci, different expression levels of *tet*(W), and/or the influence of the genetic background of the strains may account for the phenotypic differences.

Location of the *tet*(W) gene. The genetic location of *tet*(W) in the distinct species was assessed by hybridization using as a probe a 1.2-kbp internal segment of the gene obtained by PCR and labeled with digoxigenin (Roche Applied Science, Basel, Switzerland). Total and plasmid DNAs digested with the restriction enzymes EcoRI and HindIII (Roche) were hybridized using high-stringency standard conditions (hybridization at 68°C and two final washing steps in 0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% SDS at 68°C for 15 min). Figure 2 shows the results obtained with EcoRI-digested total DNA. A positive hybridization signal was obtained with all tetracycline-resistant strains and, as expected, with the susceptible strain M21. The hybridization signal appeared at a different position in each of the strains (estimated sizes, 2.5 to 16 kbp), except for the related strains *B. longum* B93 and B94 (displaying distinct RAPD-PCR patterns but isolated from the same individual). This suggests that the genetic organization of the loci might be different in each strain. The *tet*(W) locus is thought to be located on the bacterial chromosome, since only

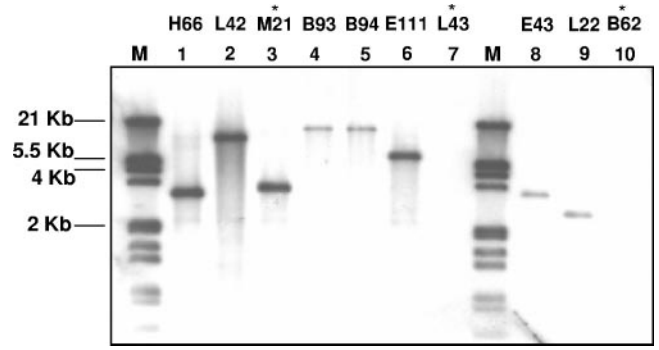

FIG. 2. Hybridization of EcoRI-digested chromosomal DNA from different *Bifidobacterium* strains using a 1.2-kbp PCR amplicon obtained with the specific primers for *tet*(W) as a probe. The code numbers of the strains are given above the lane numbers. Tetracycline-susceptible strains are asterisked. Lanes 1, 2, 3, 4, 5, 6, and 7, *Bifidobacterium longum* strains; lane 8, *Bifidobacterium animalis* E43; lanes 9 and 10, *Bifidobacterium bifidum* strains. Lanes M, molecular weight marker (digoxigenin-labeled, EcoRI- and HindIII-digested lambda DNA).

the resistant strain *B. bifidum* L71 was shown to harbor a plasmid (of around 15 kbp), and its hybridization signals did not match the position of EcoRI and HindIII plasmid fragments (data not shown).

Since the *tet*(W) gene is encoded on a conjugative transposon in *B. fibrisolvens* 1.230 (Tn*B1230*) (9, 17), primers based on transposon sequences were also synthesized and used in PCR experiments. However, no amplification products were obtained with any strain, suggesting that there are no sequences related to this mobile unit in these *Bifidobacterium* species. Further, preliminary inverse-PCR experiments indicate that *tet*(W) is inserted into a putative open reading frame encoding a permease (GenBank accession no. AE014295) of an ABC transporter system in *B. longum* NCC2705 (data not shown).

In conclusion, these and other results indicate that dominant *Bifidobacterium* species from the human GIT frequently harbor acquired tetracycline resistance encoded by a *tet*(W) gene. The fact that genes from *Bifidobacterium* showed more nucleotide changes than those from species of different genera strongly suggests independent transfer events. The spread of this gene among cattle rumen and human GIT organisms may be occurring very rapidly by unknown mechanisms that merit further research. However, preliminary conjugation experiments showed that tetracycline resistance from *B. longum* H66 and L42 did not transfer to susceptible strains (data not shown). Once acquired, genes could either be stably maintained in the absence of antibiotic selection or selected by continued exposure to antibiotics through dietary intake (5).

Nucleotide sequence accession number. The nucleotide sequence of the *tet*(W) gene and its surrounding regions from *B. longum* H66 has been deposited in GenBank (accession no. DQ060146).

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REFERENCES

1. **Barbosa, T. M., K. P. Scott, and H. J. Flint.** 1999. Evidence for recent intergeneric transfer of a new tetracycline resistance gene, *tet*(W), isolated from *Butyrivibrio fibrisolvens*, and the occurrence of *tet*(O) in ruminal bacteria. Environ. Microbiol. **1:**53–64.
2. **Chopra, I., and M. Roberts.** 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. *6***5:**232–260.
3. **Clermont, D., O. Chesneau, G. De Cespédès, and T. Horaud.** 1997. New tetracycline resistance determinants coding for ribosomal protection in streptococci and nucleotide sequence of *tet*(T) isolated from *Streptococcus pyogenes* A498. Antimicrob. Agents Chemother. **41:**112–116.
4. **Delgado, S., A. B. Flórez, and B. Mayo.** 2005. Antibiotic susceptibility of *Lactobacillus* and *Bifidobacterium* species from the human gastrointestinal tract. Curr. Microbiol. **50:**202–207.
5. **Donoghue, D. J.** 2003. Antibiotic residues in poultry tissues and eggs: human health concerns? Poult. Sci. **82:**618–621.
6. **Klare, I., C. Konstabel, S. Muller-Bertling, R. Reissbrodt, G. Huys, M. Vancanneyt, J. Swings, H. Goossens, and W. Witte.** 2005. Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, pediococci, lactococci, and bifidobacteria. Appl. Environ. Microbiol. **71:**8982–8986.
7. **Masco, L., K. Van Hoorde, E. De Brandt, J. Swings, and G. Huys.** 2006. Antimicrobial susceptibility of *Bifidobacterium* strains from humans, animals and probiotic products. J. Antimicrob. Chemother. **58:**85–94.
8. **Mättö, J., E. Malinen, M.-L. Suihko, M. Alander, A. Palva, and M. Saarela.** 2004. Genetic heterogeneity and functional properties of intestinal bifidobacteria. J. Appl. Microbiol. **97:**459–470.
9. **Melville, C. M., R. Brunel, H. J. Flint, and K. P. Scott.** 2004. The *Butyrivibrio fibrisolvens tet*(W) gene is carried on the novel conjugative transposon Tn*B1230*, which contains duplicated nitroreductase coding sequences. J. Bacteriol. **186:**3656–3659.
10. **Moubareck, C., F. Gavini, L. Vaugien, M. J. Butel, and F. Doucet-Populaire.** 2005. Antimicrobial susceptibility of bifidobacteria. J. Antimicrob. Chemother. **55:**38–44.
11. **Netherwood, T., R. Bowden, P. Harrosin, A. G. O'Donnel, D. S. Parker, and H. J. Gilbert.** 1999. Gene transfer in the gastrointestinal tract. Appl. Environ. Microbiol. **65:**5139–5141.
12. **Ouwehand, A. C., S. Salminen, and E. Isolauri.** 2002. Probiotics: an overview of beneficial effects. Antonie Leeuwenhoek **82:**279–289.
13. **Roberts, M. C.** 2005. Update on acquired tetracycline resistance genes. FEMS Microbiol. Lett. **245:**195–203.
14. **Saarela, M., G. Mogensen, R. Fonden, J. Mättö, and T. Mattila-Sandholm.** 2000. Probiotic bacteria: safety, functional and technological properties. J. Biotechnol. **84:**197–215.
15. **Salyers, A. A., A. Gupta, and Y. Wang.** 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. **12:**412–416.
16. **Scott, K. P., T. M. Barbosa, K. J. Forbes, and H. J. Flint.** 1997. High-frequency transfer of a naturally occurring chromosomal tetracycline resistance element in the ruminal anaerobe *Butyrivibrio fibrisolvens.* Appl. Environ. Microbiol. **63:**3405–3411.
17. **Scott, K. P., C. M. Melville, T. M. Barbosa, and H. J. Flint.** 2000. Occurrence of the new tetracycline resistance gene *tet*(W) in bacteria from the human gut. Antimicrob. Agents Chemother. **44:**775–777.
18. **Teuber, M., L. Meile, and F. Schwarz.** 1999. Acquired antibiotic resistance in lactic acid bacteria from food. Antonie Leeuwenhoek **76:**115–137.
19. **Ventura, M., D. van Sinderen, G. F. Fitzgerald, and R. Zink.** 2004. Insights into the taxonomy, genetics and physiology of bifidobacteria. Antonie Leeuwenhoek **86:**205–233.